STUDIES ON THE PRECIPITIN REACTION IN PLANTS

I. THE SPECIFICITY OF THE NORMAL PRECIPITIN REACTION

KENNETH S. CHESTER

Plate 42

INTRODUCTION

THE APPLICATION of the theories and methods of animal immunology to plant pathology has been a subject which up to the present has received very little attention. In large part the failure of pathologists to attack their problems from the standpoint of acquired immunity lies in the theoretical and technical obstacles inherent in a biochemical study of plant disease. Such obstacles are due in part to the differences in structure and development between plants and animals, and in part to differences in the type of infection customarily produced. Thus in plants one is dealing with organisms of indeterminate growth in comparison to the determinate growth of the higher animals, there is no circulatory system in plants closely comparable to the blood system of vertebrates, and finally the majority of plant infections are more strictly localized than are many of the animal infections.

Nevertheless, bearing in mind the close ultimate physiological relationship between the plant and the animal cell, their presumably common origin, and their essential functional similarity, it would indeed be strange to find the plant cell reacting to infections in a manner wholly distinct from that of animal cells. The work reported in the present paper was undertaken in an attempt to throw some light upon the question of the reactions of the plant cell in the presence of foreign protoplasm or its constituents, and is a continuation of the writer's previously reported studies of the precipitin reaction in plants (2).

The precipitin reaction has long been an essential technique of animal immunology. Briefly the theory of the reaction is as follows. A foreign protein is injected into the blood of a mammal. As the result of such sensitization, the injected animal acquires an immunity to the specific protein employed. After a short period of time during which the acquirement of immunity has been in progress, blood is withdrawn from the immunized animal, clarified, and pipetted against a solution of the protein originally used for injection. The immune blood now induces a precipitation of the foreign protein from solution, although the precipitating action is absent in the blood of non-immunized animals. The precipitin reaction is specific against the protein originally employed but is weak or negative against other foreign proteins. In addition to such acquired precipitating power, mammalian blood frequently possesses the ability to precipitate certain proteins with which it has never been sensitized. The substances or properties in the blood which induce such precipitation of foreign proteins with which the animal has been previously sensitized may be called *acquired precipitins,* whereas the substances or properties of the blood inducing the precipitation of proteins against which the animal has not been immunized are called *normal precipitins.*

The present paper reports a study of the normal precipitins in a number of families of woody plants. Its purposes are fourfold, namely, to determine whether in extracts of woody plants one may obtain phenomena comparable to the reactions of mammalian blood in the presence of foreign proteins, to discover whether such reactions are in any manner correlated with the systematic relationships of the plants studied, to aid in the interpretation of the earlier published data upon the precipitin reaction in plants, and finally to clarify to some extent our conception of the nature of the precipitin reaction in plants and its bearing on the processes of plant immunity.

HISTORICAL

The precipitin technique was first applied to plants by Kostoff in 1928 and 1929 (3, 4). The latter, working with a number of species of the solanaceae, observed that in numerous combinations of extracts of Solanaceous species positive reactions were obtained, whereas negative normal precipitin reactions resulted from other extract combinations. The alphabetical order in which Kostoff arranged his tables of reactions does not bring out well the significance of the reactions which he obtained, nor did he attempt to analyze them in the light of possible correlation with systematic position. A rearrangement of Kostoff's data is given in Table 6 for comparison with the results of the present study. From the standpoint of plant immunology this first paper makes a number of important contributions. The author found that after grafting two species of Solanaceae the precipitin reaction of the extracts of the intergrafted plants was markedly increased, that the increase in precipitin potency was strongest in the tissues nearest the graft union and weakened progressively with increasing distance from the graft union, and that the precipitin potency increased from the time of grafting for thirty or forty days, after which time it reached an equilibrium. In addition to the increased precipitin reaction after grafting, Kostoff also observed certain cytological changes, all of which were interpreted in terms of an acquired immunity as the result of sensitization by the foreign protein of the graft biont.

The only other study of the precipitin reaction in plants following the technique of direct testing of plant against plant is reported by Chester in 1931 (2). In this paper are considered the results of about a thousand tests in the Oleaceae. No normal precipitins were reported among the species used, but a very strong acquired precipitin reaction was obtained in *Syringa vulgaris* hybrids grafted upon *Ligustrum* species as tested against various oleaceous species. The grafted plants were displaying symptoms of disease due to an incompatibility between the lilac scion and the privet stock, and it was found that the morbid processes resulting from such incompatibility so profoundly modified the components of the lilac extract as to alter markedly the precipitin reaction. The morbidity resulting in this case from graft incompatibility was closely resembled by a morbidity from other causes, and in either case there was a marked increase of precipitin potency accompanying the appearance of morbid symptoms in the leaf.

Brief mention should be made of two other bodies of experimentation somewhat related to the work at hand. Mez and his colleagues have published extensive work dealing with an application of the precipitin reaction in animals to plant materials. The results are the basis of the Königsberg phylogenetic tree. The work of Mez and his collaborators has been published in numerous papers in Botanisches Archiv, to which the reader is referred for a complete account of the Königsberg studies. The theory of Mez' work is fundamentally different from that of the work of Kostoff and Chester, and hence need be considered no further at this time beyond remarking that the reactions obtained are the reactions of an animal injected with plant proteins and do not represent immunological reactions in which the plant is the organism acquiring an immunity.

A second body of plant immunological research deals with experiments apart from precipitin testing to determine the presence of an acquired immunity in plants subjected to disease. An excellent account of the studies on such acquired immunity in plants is to be found in the recent monograph of Carbone and Arnaudi (1). It may be said in passing that although there have been numerous conflicting reports concerning the acquirement of immunity by plants, there are a number of experiments reported by various French and Italian workers which appear to point definitely to such an acquired immunity. The paper of Carbone and Arnaudi gives a full account of these experiments, and a fairly complete bibliography of the work on acquired immunity in plants.

TECHNIQUE

In the summer of 1931, at the suggestion of Dr. Karl Sax, a set of experiments was undertaken to determine the relationships among the genera of the Pomoideae as indicated by the normal precipitin reaction. The results proved so suggestive that other subfamilies of the Rosaceae and other families of the woody plants were eventually tested to the same end. In all about five hundred reactions were performed, involving twelve genera of the Pomoideae, four genera of the Prunoideae, fifteen species of the genus *Prunus*, two other genera of the Rosaceae, nine genera of the Caprifoliaceae, eight genera of the woody Saxifragaceae, and one genus each of the Leguminosae and the Platanaceae. Except as indicated below all the experiments were performed under uniform conditions, with extracts of the same concentration, tested in the same manner, and with the employment of numerous controls. The technique has been fully described in an earlier paper (2) and only brief mention will be made of it here beyond pointing out the modifications which have been developed.

Fresh leaves of the plants to be tested were collected, weighed, washed in tap water and distilled water, dried, and ground to a fine paste in an unglazed porcelain mortar. To the paste thus obtained was added the required amount of distilled water (four times the weight of the leaves in all the experiments below) and the mixtures were placed in an electric refrigerator at 2° C for twenty-four hours. At the end of this time each mixture was filtered until crystal clear through progressively finer filters, and finally placed in an ice bath. Two to four tenths of a cubic centimeter of the liquid of greater specific gravity was next introduced into a specially-made small test-tube by means of a capillary pipette, and the second extract to be tested was pipetted above so as to form a refractive zone between the two. Readings of the reaction were taken at intervals of one, five, ten, twenty, thirty, and forty minutes, and in most of the experiments reported below readings were independently made by two observers. The utmost care was taken at every step to avoid contamination, and all instruments and glassware were cleaned with a sulphuric acid-potassium bichromate mixture for twenty-four hours followed by repeated washings in water.

The positive tests were strong and well marked. In comparison with the results earlier obtained it may be said that all the readings are minimal. There might have been justification for calling some of the plus two reactions plus three or even plus four, but an attempt was made to increase the significance of the results by using extreme caution in not overestimating the readings. A study of Plate 42 will indicate the scale employed. Figure 1 represents a negative reaction. The delimitation of the two liquids in the tube is clearly indicated, but there is no trace of a white precipitate at the zone of contact. In Figure 2 is seen a reaction indicated in the tables as a "trace" (t). Figures 3 and 4 illustrate plus one reactions, in Figure 3 the precipitate representing the ultimate intensity of the reaction between the two extracts employed while in Figure 4 the plus one reaction illustrated being merely an early stage of a reaction which after some minutes would have increased to plus two or greater. Figures 5 and 6 represent plus two reactions, Figure 6 being a later stage of the same reaction as pictured in Figure 4. Figures 5 and 6 show well the penetration of the precipitate into the lower extract in little white tortuous rootlets. Later stages of these reactions would show only a uniform cloudiness of the lower extract finally extending upward and involving all the liquid in the tube.

The only essential modification of technique that has been made in the experiments herein reported as compared with the earlier precipitin testing in plants has been that a weaker concentration of extract has been used. Heretofore the ratio of plant tissue to distilled water has been 1:2, while for the purposes of the experiments in the present paper a dilution of 1:4 was found more satisfactory. This change was made necessary by the greater percentage of water in the tissues of the plants earlier studied.

One notable advance in technique, however, has been made. There would be a number of distinct advantages if it were possible to use dried leaf tissues in place of fresh tissues. That it might be possible to obtain comparable results using dried leaves was suggested by Osborne and Wakeman's statement that spinach leaves dried at low temperatures and extracted with ether, alcohol, water, and alkaline solutions yielded results so similar, in analysis and protein extraction, that evidently the constituents of the cells are altered to only a slight degree by drying (5).

In order to test out the possibility of using dried leaves in the precipitin tests, the following experiment was performed. From the results obtained in earlier experiments three plants were so chosen that their inter-reactions would include both positive and negative results. For this purpose *Platanus acerifolia*, *Robinia fertilis*, and *Prunus Armeniaca* var. "Mikado" were arbitrarily chosen. Using fresh leaf preparations, the *Prunus* tests strongly against both the *Robinia* and the *Platanus*, whereas the latter two are negative when tested together. Leaves of these three species were collected and dried in a Riker plant press for two weeks at a temperature of about 30° C. At the end of this time the leaves were brittle and quite dry. Half of the leaves of each species were then placed in an oven at 60° C for twenty-four hours. Finally each of the six lots of leaves thus dried was ground, extracted, filtered, and tested against the other five extracts. The results were wholly satisfactory. Strong reactions were obtained between the Prunus extracts and those of the other species in every case, while the Platanus and Robinia extracts remained negative to each other. The results of this experiment are shown in Table 1.

TABLE 1. A STUDY OF THE EFFECT ON THE PRECIPITIN REACTION OF THE PREVIOUS DRYING OF THE PLANT TISSUES EMPLOYED

Explanation in the text.

	Platanus (Fresh leaves)	Platanus (Air dried)	Platanus (Oven dried)	Robinia (Fresh leaves)	Robinia (Air dried)	Robinia (Oven dried)	Prunus (Fresh leaves)	Prunus (Air dried)	Prunus (Oven dried)
Platanus acerifolia (Fresh leaves)	–			–			3		
Platanus acerifolia (Air dried leaves)		–	–		–	–		3	3
Platanus acerifolia (Oven-dried leaves)		–	–		–	–		2	2
Robinia fertilis (Fresh leaves)	–			–			3		
Robina fertilis (Air dried leaves)		–	–		–	–		2	2
Robinia fertilis (Oven dried leaves)		–	–		–	–		2	2
Prunus Armeniaca (Fresh leaves)	3			3			–		
Prunus Armeniaca (Air dried leaves)		3	2		2	2		–	–
Prunus Armeniaca (Oven dried leaves)		3	2		2	2		–	–

It will be seen that there is a slightly higher reaction accompanying the use of air dried leaves in one case, and although the distinction was too fine to be indicated by the difference of a plus sign, it should be noted that the extracts from air dried leaves always tested slightly more strongly than those from the oven-dried leaves. The reactions as a whole were slightly weaker than those where fresh leaves were used, but the difference is not considered significant. It was impossible to equalize the concentration of the two types of extract with certainty, since the percentage of water in the fresh leaves was not known. In the case of the fresh leaves the weight ratio of leaf tissue to water of extraction was as 1 : 4, while with the dried leaves the corresponding ratio selected was 1 : 10.

The ability to utilize dried leaves in precipitin testing has, as has been indicated, a number of advantages. It is thus possible to continue work with the deciduous woody plants throughout the winter; moreover one may work with specimens shipped in a dried condition from all parts of the globe; it is possible to employ dried herbarium specimens in testing; manipulation of dried material is much easier than of fresh leaves with especial respect to grinding; the extracts made with dried leaves filter much more readily than those made with fresh leaves; and finally adjustment of the water concentration of the extracts may be made with much greater accuracy. The fact that experiments may be performed with essentially the same results whether one uses dried or fresh leaves does not argue against the hypothesis of the protein nature of the reaction according to the evidence of Osborne and Wakeman as cited above.

EXPERIMENTAL DATA

The actual experimental results are shown in the following tables (Tables 2–6). With them is included for comparison a synopsis of the results obtained by Kostoff on the Solanaceae, rearranged according to the systematic position of the genera.

READING AND INTERPRETATION OF EXPERIMENTAL DATA

A word of interpretation is a necessary preliminary to a study of the tables. In contrast to the work of Mez and others, an increasing positive reaction here indicates an increasing degree of divergence from the type. The negative reaction (–) appears to have a double significance. A study of Table 2 will bring this out. In the upper left-hand corner there is a block of negative reactions indicative of the homogeneity of the Pomoideae. Traces of reactions appear in a few instances, but for the main part the results are uniformly

TABLE 2. NORMAL PRECIPITIN REACTIONS IN THE POMOIDEAE AND RELATED GENERA

Explanation in the text.

	Spiraea virginiana	Cotoneaster acutifolia	Mespilus germanica	Pyracantha coccinea	Crataegus punctata	Sorbus Aucuparia	Aronia melanocarpa	Photinia villosa	Stranvaesia Davidiana	Chaenomeles lagenaria	Malus prunifolia	Malus Tschonoskii	Pyrus nivalis	Amelanchier oblongifolia	Rosa rugosa	Prunus Armeniaca	Platanus acerifolia	Robinia fertilis	Philadelphus grandiflorus
Spiraea virginiana	–			–	–			t			–	–			1	2	t	t	t
Cotoneaster acutifolia		–	–	t	–	–	t	t	t	–	t		–	t					
Mespilus germanica		–	–	–	2	–	–	–	–	–	–		–	1					
Pyracantha coccinea	–	t	–	–	–	–	–	–	–	t	–	t	–	–	2	2	–	–	–
Crataegus punctata	–	–	2	–	–	–	–	–	–	–	–	–	–	–	t	t	t	t	t
Sorbus Aucuparia		–	–	–	–	–	–	–	–	–	–		–	–					
Aronia melanocarpa		t	–	–	–	–	–	–	–	–	–		–	–					
Photinia villosa	t	t	–	–	–	–	–	–	–	–	–	t	–	–	1	2	–	–	–
Stranvaesia Davidiana		t	–	–	–	–	–	–	–	–	–		–	–					
Chaenomeles lagenaria		–	–	t	–	–	–	–	–	–	t		–	–					
Malus prunifolia	–	t	–	–	–	–	–	–	–	t	–	t	–	–	2	2	–	–	–
Malus Tschonoskii	–			t	–			t			t	–			–	2	t	–	–
Pyrus nivalis		–	–	–	–	–	–	–	–	–	–		–	–					
Amelanchier oblongifolia		t	1	–	–	–	–	–	–	–	–		–	–					
Rosa rugosa	1			2	t			1			2	–			–	2	2	1	2
Prunus Armeniaca	2			2	t			2			2	2			2	–	3	3	3
Platanus acerifolia	t			–	t			–			–	t			2	3	–	–	–
Robinia fertilis	t			–	t			–			–	–			1	3	–	–	–
Philadelphus grandiflorus	t			–	t			–			–	–			2	3	–	–	–

negative. The reaction steadily increases as one passes farther from the Pomoideae, beginning with *Spiraea* which is very closely allied to the Pomoideae, passing to Rosa which is somewhat more distantly related to the Pomoideae, and finally reaching *Prunus* with the strongest reaction of all, as might well be expected since of all the subfamilies of the Rosaceae the Prunoideae are farthest removed from the Pomoideae. As we pass outside the family, however, the reaction again diminishes. This phenomenon has been observed in a number of cases (cf. Tables 3 and 4). In other words, reasoning from the data thus far available, there appears to be a degree of divergence which results in a maximum reaction. If this degree of divergence be either exceeded or decreased the reaction appears to diminish in intensity. One is immediately struck by the analogy to the "zone phenomenon" in animal immunology, where there is an optimum concentration for reaction, and where if this optimum be passed in either direction the reaction diminishes. There is one significant difference in the plant phenomenon, however, namely that whereas in the zone phenomenon one has to do solely with a quantitative optimum, in the case at hand it appears to be a qualitative difference which determines the optimum of reaction. Thus in the normal plant precipitin reactions the optimum condition for reaction, according to the hypothesis above, implies a qualitative difference in the reacting substances which must be neither too great nor too small. Hence, one would infer that a negative reaction might imply a very close relationship or a very great divergence, whereas a positive reaction would involve a definite degree of affinity. It would thus be impossible from a single reaction to judge the degree of affinity of two plants; such judgement could only follow from a consideration of the reactions of the plant in question in relation to the reactions of a number of related species. As a case in point, the first reactions performed with *Prunus* involved eight species. From the reactions thus obtained it was very difficult to arrive at a logical interpretation. Seven additional species of *Prunus* were chosen and tested against the original species and against one another. The result was that the seven additional species offered connecting links and transitions of such value that the fifteen species at once fell into a logical order, in fact an order which with but one exception agrees with the order of arrangement accepted by present-day taxonomists of the genus.

One other fact must also be taken into consideration. As is well known to taxonomists, groups of plants vary among one another in *variability*. A given character which may be very uniform in one heterogeneous group may be very variable in another more homo-

TABLE 3. NORMAL PRECIPITIN REACTIONS IN THE PRUNOIDEAE

Explanation in the text.

	Prunus spinosa	Prunus insititia	Prunus domestica	Prunus cerasifera	P. salicina × Simonii	Prunus maritima	Prunus hortulana	Prunus Armeniaca	Prunus Persica	Prunus Davidiana	Prunus pumila	Prunus serrulata	Prunus avium	Prunus serotina	Prunus Padus	Osmaronia cerasiformis	Prinsepia sinensis	Maddenia hypoleuca
Prunus spinosa	–	–	–	–	1	1	1	2	–	2	t	2	2	2	2	2	2	2
Prunus insititia	–	–	–	–	1	1	1	1	2	2				2	2			
Prunus domestica	–	–	–	–	–	–	2	1	t	2				2	2			
Prunus cerasifera	–	–	–	–	2	2	1	–	–	2				2	2			
Prunus salicina × Simonii	1	1	–	2	–	–	2											
Prunus maritima	1	1	–	2	–	–	1	2	2	2				2	2			
Prunus hortulana	1	1	2	1	2	1	–	2	t	–	–	–	–	–	–	–	–	–
Prunus Armeniaca	2	1	1	–		2	2	–	–	2	2	2	2	2	3	2	2	2
Prunus Persica	–	2	t	–		2	t	–	–	2				1	2			
Prunus Davidiana	2	2	2	2		2	–	2	2	–	t	1	2	–	–	–	–	–
Prunus pumila	t						–	2		t	–	–	–	–		t	–	–
Prunus serrulata	2						–	2		1	–	–	–	1		1	t	–
Prunus avium	2						–	2		2	–	–	–	1		2	2	–
Prunus serotina	2	2	2	2		2	–	2	1	–	–	1	1	–	–	1	–	–
Prunus Padus	2	2	2	2		2	–	3	2	–				–	–			
Osmaronia cerasiformis	2						–	2		–	t	1	2	1		–	–	–
Prinsepia sinensis	2						–	2		–	–	t	2	–		–	–	–
Maddenia hypoleuca	2						–	2		–	–	–	–	–		–	–	–

geneous group. Such is the case with the precipitin reaction. Because one finds the Caprifoliaceae to be rather uniformly negative among themselves whereas the genus *Prunus* exhibits a high degree of variability does not necessarily imply that the Caprifoliaceae as a group are more homogeneous than the genus *Prunus*. *Prunus* may be homogeneous in many characters, but in its precipitin reaction it exhibits a remarkable variability. Whatever the character chosen as fundamental in a taxonomic study, the same phenomenon occurs. Each group must be judged by itself and its phylogenetic relationships must at present necessarily be determined by no one character but by the bulk of evidence yielded by all characters, morphological, anatomical, cytological, genetic, and physiological.

TABLE 4. NORMAL PRECIPITIN REACTIONS IN THE SAXIFRAGACEAE

Explanation in the text.

	Philadelphus grandiflorus	Fendlera Wrightii	Jamesia americana	Deutzia scabra plena	Hydrangea paniculata	Schizophragma hydrangeoides	Itea virginiana	Ribes petraeum	Prunus Armeniaca	Robinia fertilis	Photinia villosa	Platanus acerifolia
Philadelphus grandiflorus	–	–	–	–	–	–	t	2	2	1	–	t
Fendlera Wrightii	–	–	3	1	–	–	2	3	3	2	2	1
Jamesia americana	–	3	–	–	–	t	–	2	3	t	1	–
Deutzia scabra plena	–	1	–	–	–	t	–	1	t	2	–	–
Hydrangea paniculata	–	–	–	–	–	1	t	2	2	2	–	–
Schizophragma hydrangeoides	–	–	t	t	1	–	1	2	3	2	–	t
Itea virginiana	t	2	–	–	t	1	–	2	2	1	1	t
Ribes petraeum	2	3	2	1	2	2	2	–	–	2	3	2
Prunus Armeniaca	2	3	3	t	2	3	2	–	–	3	2	3
Robinia fertilis	1	2	t	2	2	2	1	2	3	–	–	–
Photinia villosa	–	2	1	–	–	–	1	3	2	–	–	–
Platanus acerifolia	t	1	–	–	–	t	t	2	3	–	–	–

A COMPARISON BETWEEN THE SYSTEMATIC RELATIONSHIPS INDICATED BY THE EXPERIMENTS AND THE RELATIONSHIPS INDICATED BY MORPHOLOGICAL TAXONOMY

In projecting the normal precipitin reaction in plants as an aid in systematic studies of the plant groups it is of utmost importance to ascertain the degree of correlation which exists between the relationships as indicated by the reaction and those accepted by modern taxonomists. As has been pointed out an exact parallel with morphological systems is neither found nor to be expected.

TABLE 5. NORMAL PRECIPITIN REACTIONS IN THE CAPRIFOLIACEAE

Explanation in the text.

	Sambucus canadensis	Viburnum cassinoides	Symphoricarpus mollis	Dipelta ventricosa	Abelia Zanderi	Linnaea borealis	Kolkwitzia amabilis	Diervilla florida	Lonicera Myrtillus	Pyracantha coccinea	Deutzia scabra plena	Platanus acerifolia
Sambucus canadensis	–	–	–	–	–	–	–	–	–	–	–	–
Viburnum cassinoides	–	–	–	1	1	–	–	–	–	–	–	–
Symphoricarpus mollis	–	–	–	t	–	–	–	–	–	–	–	–
Dipelta ventricosa	–	1	t	–	–	–	1	–	–	–	t	–
Abelia Zanderi	–	1	–	–	–	–	–	–	–	1	t	–
Linnaea borealis	–	–	–	–	–	–	–	–	–	–	–	–
Kolkwitzia amabilis	–	–	–	1	–	–	–	–	–	–	–	–
Diervilla florida	–	–	–	–	–	–	–	–	–	–	–	–
Lonicera Myrtillus	–	–	–	–	–	–	–	–	–	–	–	–
Pyracantha coccinea	–	–	–	–	1	–	–	–	–	–	–	–
Deutzia scabra plena	–	–	–	t	t	–	–	–	–	–	–	–
Platanus acerifolia	–	–	–	–	–	–	–	–	–	–	–	–

However, the value of the reaction as a systematic tool depends upon a general conformity with our present knowledge of the main trends in taxonomy, and the present section will hence be devoted to a consideration of the question of whether such general conformity does exist. The discussion of the results of the precipitin experiments in relation to the systematic position of the plants tested must necessarily be confined to the broader, more pronounced re-

TABLE 6. KOSTOFF'S RECORD OF NORMAL PRECIPITIN REACTIONS IN THE SOLANACEAE, WITH THE GENERA ARRANGED ACCORDING TO THE SYSTEMATIC TREATMENT OF ENGLER-GILG—Explanation in the text.

	Lycium barbarum	Solanum nigrum	Solanum Dulcamara	Solanum Melongena	Solanum Lycopersicum	Solanum tuberosum	Capsicum pyramidale	Physalis peruviana	Datura Wrightii	Datura ferox	Nicotiana suaveolens	Nicotiana Langsdorfii	Nicotiana Tabacum	N. rustica × Tabacum	Nicotiana rustica	Nicotiana alata	Nicotiana paniculata	Nicotiana glauca	Nicotiana Rusbyi	Petunia violacea	Salpiglossis sinuata
Lycium barbarum	–	–	3	t	–	–	2	–	2	2	t	–	t	–	–	–	–	t	–	2	–
Solanum nigrum	–	–	–	–	–	–?	4	2	4		t	–	–	–	2	–	–	1	3	–	3
Solanum Dulcamara	3	–	–	–			4	4	4						–			–			4
Solanum Melongena	t	–	–	–	–		2	–	4		–	–	–	–	–	–	–	–	1	–	3
Solanum Lycopersicum	–	–		–	–	–	1		4	1	–		–	–	–	–	–	–	1	–	3
Solanum tuberosum	–	?			–	–	–	–	4			2	–	–	t	2	–	–	–		
Capsicum pyramidale	2	4	4	2	1	–	–	–	t		–	2	1	–	1	1	1	t	t	–	–
Physalis peruviana	–	2	4	–		–	–	–							3			2			–
Datura Wrightii	2	4	4	4	4	4	t		–				?								–
Datura ferox	2				1					–			2?			t					
Nicotiana suaveolens	t	t		–	–		–				–	–	1	–	–	–	–	–	3	–	–
Nicotiana Langsdorfii	–	–		–		2	2				–	–	–	–	–	–	–	–	3	1	–
Nicotiana Tabacum	t	–		–	–	–	1		2?	?	1	–	–	–	–	–	t	–	2	–	4
Nicotiana rustica × Tabacum	–	–		–	–	–	–				–	–	–	–	–	–	–	–	1	–	–
Nicotiana rustica	–	2	–	–	–	t	1	3			–	–	–	–	–	–	–	–	1	–	4
Nicotiana alata	–	–		–	–	2	1			t	–	–	–	–	–	–	–	–	1	–	–
Nicotiana paniculata	–	–		–	–	–	1				–	–	t	–	–	–	–	–	t	–	t
Nicotiana glauca	t	1	–	–	–	–	t	2			–	–	–	–	–	–	–	–	–	–	3
Nicotiana Rusbyi	–	3		1	1	–	t				3	3	2	1	1	1	t	–	–	3	–
Petunia violacea	2	–		–	–		–				–	1	–	–	–	–	–	–	3	–	–
Salpiglossis sinuata	–	3	4	3	3		–	–	–		–	–	4	–	4	–	t	3	–	–	–

lationships of the groups, since a detailed analysis of the value of each reaction would be possible only to a monographer of the respective groups equipped with a very extensive knowledge of the evidences of relationship yielded by several methods of approach.

Referring to Table 2 one is first impressed by the relative homogeneity of the Pomoideae. This point is in conformity with the findings of taxonomists in general that the Pomoideae as a whole represent a well marked and uniform subfamily. The one strong reaction indicated, that of *Mespilus* plus *Crataegus*, is susceptible to a somewhat different explanation than any of the other reactions indicated in this paper. *Mespilus* and *Crataegus* are assumed to be very closely allied. One would expect, if this be true, a negative reaction. The plant of *Mespilus* chosen, however, had been propagated by grafting upon *Crataegus*, and the reaction may very well represent a modification of the normal immunity. Passing outside the subfamily, the Spiraeoideae as represented by *Spiraea virginiana* show a close affinity to the Pomoideae, the Rosoideae would be somewhat farther removed, while the Prunoideae appear to be farthest removed of all from the Pomoideae. Such an arrangement as indicated by the precipitin reaction conforms to the opinion of Rehder and others that the Spiraeoideae are relatively close to the Pomoideae on the basis of morphology, while the Rosoideae and the Pomoideae are more divergent in the order named. It may be claimed that such a comparison as the above, based upon a single species of each subfamily, is unwarranted. It is indeed possible that a selection of numerous species of each subfamily would show variation among themselves in their reactivity toward the Pomoideae, but in the absence of more extensive data upon this point one may merely note that the species chosen at random do show such a progressive removal from the Pomoideae as would be expected from a knowledge of the taxonomy of the group involved. The other three genera chosen for testing against the Pomoideae are all hypothetically too far removed from that subfamily to show marked reactions.

Passing to the Prunoideae (Table 3) one is first impressed by the remarkable variability evidenced as compared with the Pomoideae. The Prunoideae as a whole are a much more complex group than the Pomoideae and such greater variability might well be anticipated. *Prunus spinosa* and *P. insititia* exhibit reactions very similar in general, conforming with their close morphological relationship. Moreover the Euprunus group (represented here by *P. spinosa*, *P. insititia*, *P. domestica*, *P. cerasifera*, and *P. salicina* × *P. Simonii*) as a whole are practically negative among one another but positive to the remainder of the genus. The transition from the Euprunus group to *P. maritima* in *P. salicina* × *P. Simonii* is readily interpreted in the light of the close relationship of *P. maritima* to the Plums. The reactions of *P. Armeniaca*, *P. Persica*, and *P. Davidiana* in general are somewhat less uniform, although a relationship between *P. Armeniaca* and *P. Persica* is apparent. *P. Davidiana* diverges curiously, a point which might well be of interest to a student of the group. The Cherries as a group, *P. pumila*, *P. serrulata*, and *P. avium*, are well set off from the preceding members of the genus and are mutually negative as would be anticipated. Finally the Padus group, *P. serotina* and *P. Padus*, are mutually negative and show their only affinities with the Cherries, which is in accordance with taxonomic findings.

Certain exceptional reactions in the genus are of interest. Among these should be mentioned the aberrance of *P. hortulana* and the distinction among the Peaches already indicated. That *P. Persica* should react positively with *P. Davidiana* is certainly worthy of note. Finally as one passes outside the genus the reactions diminish in accordance with the hypothesis previously pointed out.

In the Saxifragaceae (Table 4) occur a number of interesting reactions. As a whole the group of reactions exhibits a parallel with the taxonomic treatments. *Ribes* is by far the most aberrant genus, as would be expected, with *Itea* following closely after. The strong reaction of *Fendlera* and *Jamesia* is worthy of comment, as the two genera are considered to be rather closely allied, while one is somewhat surprised at the slight divergence of *Hydrangea* and *Schizophragma*.

The Caprifoliaceae (Table 5) as a whole are rather disappointing from the standpoint of the precipitin reaction. The group is rather heterogeneous and a number of strong reactions were anticipated. However, as has been already suggested, the groups selected would be expected to vary from one another in variability and the technique would necessarily be of more value in some groups than in others. *Sambucus* is taxonomically very distinct from the rest of the family. Hypothetically its negative reactions may well indicate too great a divergence to result in positive tests. *Viburnum* of all the genera considered is most reactive, which accords with its intermediate position between *Sambucus* and the rest of the family. The *Dipelta-Kolkwitzia* reaction is puzzling, but *Dipelta* is rather aberrant and its aberrance as expressed by the precipitin reaction may well be greater than that which would be expected from a study of morphological structure.

Finally a consideration of the Solanaceae (Table 6) is of interest.

It should be recognized at the start that the reactions of the Solanaceae as here indicated were performed by a different worker, under different experimental conditions, and hence may not be strictly comparable to the results in the other tables. Attention is first directed to the reactions of the genus *Nicotiana*. As a whole the genus is very uniform, *N. Rusbyi* alone being divergent. The negative reactions of the latter with the other twelve-chromosome members of the genus, *N. glauca* and *N. paniculata*, may well be of significance, but its strong reactions with the remainder of the genus are problematical. The species of *Solanum* are uniformly negative with one another and thereby stand apart as a homogeneous group fairly closely allied, according to the reactions, with *Nicotiana*, *Capsicum* and *Physalis*, generally accepted as very close taxonomically, are mutually negative, and on the whole rather strongly set off from the neighboring genera. *Salpiglossis* and *Petunia* exhibit a like relationship, being mutually negative but distinct from all the other genera. Of all the groups considered the Solanaceae is probably the most complex and poorly defined. It is likely that many of the species are the products of a reticular phylogeny, and if this be true, the reactions are of particular interest. It may very well be that the group is so large that some of the more peripheral negative reactions represent divergence rather than affinity, but the positive reactions on the whole show a reasonable correlation with what is known of the relationships of the family.

It is of value at this point to consider the theoretical results of a series of precipitin tests in a circumscribed group in order to form a clearer conception of the correlation between the theoretical expectation and the experimental yield. Assuming a group of five species capable of being arranged in a linear series, each of the assumed species being equidistant in all characters from the two adjacent species, the reactions obtained would theoretically be of the general form:

	A	B	C	D	E
A	–	t	1	2	3
B	t	–	t	1	2
C	1	t	–	t	1
D	2	1	t	–	t
E	3	2	1	t	–

The significant facts to draw from such a theoretical table are that in the reactions of such an ideal group there would be a concentration of the strongest reactions in the upper right-hand and lower left-hand corners, that there would be a path of negative reactions extending along the opposite diagonal, and that the reactions would progressively increase in strength as one passed from any point on the diagonal toward the opposite corners of the table. The situation pictured, however, would rarely if ever obtain, since circumscribed groups of species or genera customarily tend to fall into a number of subgroups, each subgroup being relatively homogeneous and distinct as a body from the other subgroups. Selecting the genus *Prunus*, since that is the group which has been most thoroughly investigated from the standpoint of the precipitin reaction, one may plot a theoretical expectation for a group of species conforming in main outlines to this genus. Rehder's treatment of the genus (6) would divide the species considered in the following manner. The first eight species indicated in the table would fall into a single subgenus Prunophora. This group of eight may be further subdivided into three subgroups, a first subgroup (Euprunus) including *P. spinosa* to *P. salicina* × *P. Simonii*, a second subgroup represented by *P. maritima* and *P. hortulana* (Prunocerasus), and a third subgroup (Armeniaca) containing *P. Armeniaca*. *P. Persica* and *P. Davidiana* would represent the second subgenus (Amygdalus), *P. pumila*, *P. serrulata*, and *P. avium* the third (Cerasus), and *P. serotina* and *P. Padus* the fourth (Padus). In other words the species considered may be divided into six groups, consisting of 5, 2, 1, 2, 3, and 2 species respectively. If the theoretical yield of a body of fifteen species so divided be plotted, the resulting chart would have the general form of Table 7.

By comparing this theoretical arrangement of reactions with the actual reactions obtained in *Prunus* (Table 3) one immediately sees the resemblance. In the upper left-hand corner of each there is a block of negative signs indicative of the close relationship of the Euprunus group. The hybrid "Wickson" Plum (*P. salicina* × *P. Simonii*) alone begins to diverge from the Euprunus type. The path of negative reactions from upper left to lower right is well marked on both tables as is also the concentration of the more powerful reactions in the opposite corners. It will be observed that in the table of Prunus reactions the arrangement of Rehder was strictly followed. Several factors tend to cause some degree of divergence from the ideal grouping. Thus the ideal table assumes that the subgroups are perfectly homogeneous, that they are all equidistant from their adjacent subgroups, and that they can be arranged in a linear series. In actual practice none of these conditions obtains. The real relationships between the fifteen selected species of *Prunus* could probably be represented only by a three-dimensional figure in part dendritic in form and in part reticular. Convergence undoubtedly accounts for some of the similarities. In practice these several factors lead to a certain amount of variation from the ideal distribution, but that the experimental chart in its general features conform strikingly to the ideal chart affords definite evidence of the value of the method in taxonomy and of the specificity of the reaction in immunology.

TABLE 7. THEORETICAL EXPECTATION OF PRECIPITIN REACTIONS IN AN IDEAL GROUP OF THE GENERAL STRUCTURE OF THE GROUP OF PRUNUS SPECIES TESTED (TABLE 3)

Explanation in the text.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
A	–	–	–	–	–	t	t	1	2	2	2	2	2	2	2
B	–	–	–	–	–	t	t	1	2	2	2	2	2	2	2
C	–	–	–	–	–	t	t	1	2	2	2	2	2	2	2
D	–	–	–	–	–	t	t	1	2	2	2	2	2	2	2
E	–	–	–	–	–	t	t	1	2	2	2	2	2	2	2
F	t	t	t	t	t	–	–	t	1	1	2	2	2	2	2
G	t	t	t	t	t	–	–	t	1	1	2	2	2	2	2
H	1	1	1	1	1	t	t	–	t	t	1	1	1	2	2
I	2	2	2	2	2	1	1	t	–	–	t	t	t	1	1
J	2	2	2	2	2	1	1	t	–	–	t	t	t	1	1
K	2	2	2	2	2	2	2	1	t	t	–	–	–	t	t
L	2	2	2	2	2	2	2	1	t	t	–	–	–	t	t
M	2	2	2	2	2	2	2	1	t	t	–	–	–	t	t
N	2	2	2	2	2	2	2	2	1	1	t	t	t	–	–
O	2	2	2	2	2	2	2	2	1	1	t	t	t	–	–

Finally lest it be asserted that the distribution of reactions obtained might well have been fortuitous, one may compute the probability that seventy-five reactions distributed at random might as closely resemble the ideal distribution as do the experimental results in Table 3. It will be found that the probability of such a distribution by chance alone is exceedingly small.

From the comparison which has been made between the relationships indicated by the reaction and those indicated by recent taxonomic studies, one can now reach a conclusion as to the systematic value of the reaction. It has been seen that on the whole the reactions are expressive of the same broad phylogenetic trends as are indicated by the conventional taxonomic methods. Incompatibilities do exist, although for the main part they are minor as compared with the main features of agreement. That there are such incompatibilities is no more characteristic of the precipitin technique than of other techniques of phylogenetic investigation, and the precipitin reaction thus offers to the intensive monographer at the same time a technique for shedding light upon his more debatable relationships, and a challenge in interpretation.

EVIDENCE AS TO THE NATURE OF THE REACTION

The taxonomic value of the reaction having been pointed out, it is desirable at this point to consider the evidence yielded by the experiments in hand as to the nature of the reaction. The precipitin technique may be interpreted either as a purely physical phenomenon, as a reaction of some non-specific chemical compound of the extracts, or finally as a reaction of the specific proteins of the extracts. It is important to know which of these interpretations is applicable not only in determining the stress which is to be laid upon the reaction from the phylogenetic standpoint, but also in evaluating the work which has been done in applying the technique to a study of plant immunity. In this, as in most other biological problems, the solution depends upon the evidence yielded by two main lines of investigation, the descriptive and the analytical. The present paper treats of the precipitin reaction from the descriptive approach. Before a final conclusion is reached as to the nature of the reaction and its consequent value in taxonomy, immunology, and parasitology, the analytical method must be applied. The reaction must be subjected to an intensive study employing the techniques of biochemistry. Such a study is now in progress, and upon the results will depend the final interpretation of the precipitin reaction in plants. Meanwhile, however, it is instructive briefly to point out the facts which have been yielded by the descriptive mode of attack as having a bearing upon the solution of the problem as to the nature of the reaction.

With regard to the hypothesis that the reaction may be due to purely physical variables, the evidence at present indicates that such is not the case. Kostoff (4), in an extensive series of pH determinations in the solanaceous extracts with which he worked, showed that the precipitin reaction bears no relation to the pH of the extracts employed. The temperature of the extracts has been so fully controlled that it could not conceivably function as a cause for the precipitation. That the concentration of the extracts alone does not account for the reaction is evidenced by the facts that minor dilutions do not appreciably affect the reaction and that the progressive dilution of one or both of the extracts merely progressively weakens the precipitation. Furthermore, it is immaterial which extract be pipetted above the other in testing. The relative position of the extracts may be reversed without affecting the potency of the reaction. It is difficult to conceive of any other purely physical variable which could be responsible for the reaction.

With a purely physical explanation of the reaction thus eliminated one is forced to conclude that the precipitation is due either to the action of some non-specific chemical component of the extracts, or to a relatively specific substance of enzymatic nature, or to the highly specific proteins. It would exceed the bounds of scientific caution at present to attempt to ascribe the precipitin reaction to one of these three groups of substances. The specificity of the reaction and its general agreement with the complexity of the taxonomic treatments inclines one to the belief that specific proteins are involved. On the other hand, some of the reactions, such as those of *Prunus*, *Ribes*, and *Robinia* in Table 4 lead one to the suspicion that possibly some non-specific compound is acting in a rather complex fashion to produce the precipitates. As has been indicated above the solution of the problem of the nature of the reaction ultimately depends upon a chemical analysis of the reaction and it is more prudent to suspend decision until the results of the biochemical investigation are available.

PRACTICAL APPLICATION OF THE PRECIPITIN REACTION

A word should be introduced at this point regarding the practical application of the precipitin technique in systematic studies of plant groups. It has been shown in the preceding pages that the reaction bears a definite relation to the systematic position of the plants studied, and that this relation is too well correlated with taxonomic positions to be accidental. That we have in the precipitin reaction an additional taxonomic tool is apparent. The technique is somewhat tedious in application and as a result its employment will necessarily be limited to such debatable phylogenetic problems as must be attacked from every available angle. If the reaction is truly protein in nature it is conceivable that the evidence obtained is more fundamental than that yielded by a study of secondary characters. In its present state of development the precipitin technique is necessarily limited to small groups and will prove to be much more sensitive in some groups than in others. A more explicit statement of the value of the reaction in systematic studies awaits a final determination of the nature of the reaction from the biochemical standpoint.

The method of direct precipitin testing in plants is not comparable to the technique involving the sensitization of animals with plant extracts. If proteins are involved the direct technique as outlined in the present paper has a number of advantages over the older technique. Among these advantages are the greater simplicity of application, the freedom from errors produced by the variability in the experimental animals, and the absence of more fundamental errors due to the fact that an animal sensitized to a given species of plant will react positively to the extract from a widely separated species containing an homologous protein. For as Wells and Osborne have pointed out (7) the same protein may occur in widely separated species, accompanying the proteins upon which depend the specificity of the respective species. The direct precipitin method reveals only the *differences* in reactivity of the extracts, homologous reactive substances being neutralized in the process of extraction. On the other hand, in its present form the direct precipitin technique is certainly much less sensitive than the blood technique, although the results as indicated in Tables 2–6 demonstrate that an application is useful in limited plant groups.

In conclusion it may be said that the positive reactions yielded by plant extracts in the presence of foreign extracts is indicative of a phase of immunity against disease which has as yet received scant attention in plant pathology. It is well known that susceptibility and immunity in plants in many cases depend on more than mechanical obstructions to the invading organism, the presence of toxins, or the absence of conditions vital to the development of the parasite. An application of the principle of incompatibility as developed above may well afford an answer to some of the heretofore inexplicable problems of the parasitology of plants.

SUMMARY

1. The present paper describes a series of tests of the direct normal precipitin reactions in a number of families of woody plants for the purpose of determining the specificity of the reaction and its consequent relation to applications of the method in phylogeny and immunology.

2. A marked advance in technique, namely the ability to utilize

dried leaf tissues in the tests, is described, and its advantages over the use of fresh tissues are pointed out.

3. In general the results of the experiments performed indicate a clear-cut parallel betweeen the systematic relationships as indicated by the precipitin reaction and the main trends of relationship as indicated by the conventional methods of taxonomy. The divergences from such a parallel are no greater than are found in the comparison of the relationships indicated by any two systematic methods. Uniform groups of plants are in general characterized by homogeneity in reaction and absence of mutual reactivity, whereas the reactions steadily increase as one passes farther from the type originally selected, reaching a maximum at a given distance from the type, and then again disappearing as the divergence of the plants hypothetically becomes too great to be expressed in reaction.

4. An analysis is made of the nature of the reaction on the basis of the evidence yielded by the study of normal precipitins. The hypothesis that the reaction may be due to purely physical variables is shown to be untenable, and attention is hence directed to the influence of specific components of the extracts studied.

5. The parallel obtained between the results of the precipitin tests and the accepted systematic relationships indicates the value of an application of the precipitin technique to intensive studies of circumscribed plant groups both in confirming the results of systematic investigations employing other methods of attack and in shedding light upon the more debatable relationships between certain genera and species.

6. The specificity manifested by the normal precipitin reaction affords definite indication of the value of an application of the technique in a study of the nature of immunity to disease in plants.

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PATHOLOGICAL LABORATORY, ARNOLD ARBORETUM,
HARVARD UNIVERSITY,
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